REMARKS

The Present Invention and Pending Claims

Claims 28-34, 37-40, 43-51, 54, and 56-58 are pending and are directed to a method of creating a transgenic non-human animal containing a gene encoding an expressible dominant negative protein to a naturally occurring cellular protein (claims 28-34, 37, and 57), a transgenic non-human mammal (claims 38-40, 43-51, 56, and 58), and a method of producing a transgenic non-human mammal capable of expressing a protein which has a biological activity of an acidic dominant negative to a cellular protein (claim 54).

Summary of the Office Action

The Office has rejected claims 28-34, 37-40, 43-51, 54, and 56-58 under 35 U.S.C. § 112, first paragraph, as allegedly lacking written description. The Office also has rejected claims 28-34, 37-40, 43-51, 54, and 56-58 under 35 U.S.C. § 112, first paragraph, as allegedly lacking enablement. Reconsideration of these objections and rejections is hereby requested.

Examiner Interview

Applicants thank Examiner Quang Nguyen for the telephonic interview, which took place on August 10, 2004, with Applicants' representative, Rachel J. Mejdrich. During the interview, Applicants' representative discussed the outstanding rejections with Examiner Nguyen, as well as clarifying the technical issues cited in the outstanding rejections.

Discussion of the Rejections

The Office has rejected the pending claims for allegedly lacking written description and enablement. These rejections are traversed for the following reasons.

A. Written Description Requirement

The Office contends that a skilled artisan cannot envision the detailed structure, particularly any useful phenotype associated with any transgenic non-human animal/mammal whose genome contains a gene encoding an expressible dominant negative protein to a Fos protein or a method for producing the transgenic non-human animal/mammal (see Office Action, page 4). Additionally, the Office contends that the specification does not provide a representative number of species of a transgenic non-human animal/mammal containing a gene encoding an expressible dominant negative protein to a Fos protein, especially in view of the unpredictability of the transgenic art at the filing date of the application (see Office Action, page 4).

The pending claims are directed to a method of creating a transgenic non-human animal (e.g., mouse) containing a gene encoding an expressible dominant negative protein to a naturally occurring cellular protein, a transgenic non-human mammal (e.g., mouse), and a method of producing a transgenic non-human mammal (e.g., mouse). The specification teaches that the transgenic non-human animal/mammal (e.g., mouse) of the invention has a viable phenotype, and can be used to evaluate and assess the *in vivo* effects of the expression of a dominant negative protein (e.g., a dominant negative protein to Fos) (e.g., in specific tissues and/or in a multi-stage carcinogenesis model), for rational drug design, and for testing supplemental treatments, drugs, and therapies for use with the dominant negative protein (e.g., a dominant negative protein to Fos) (see, e.g., pages 7-8, paragraph [0022], and the Rule 132 Declaration of Charles R. Vinson, Ph.D. submitted with the "Response to Office Action" dated March 3, 2004). Supplying an inactivating or inhibitory function by the dominant negative protein (e.g., a dominant negative protein to Fos) leads to and/or causes the suppression of neoplastic growth in a variety of cell types, especially those cells that have been growth-altered (e.g., in a multi-stage carcinogenesis model) (see, e.g., page 37, paragraph [0090] and the Rule 132 Declaration of Charles R. Vinson, Ph.D.). Accordingly, the specification describes multiple phenotypes associated with dominant negative nucleic acid binding proteins (e.g., a dominant negative protein to Fos) including decreased cell growth, suppression of neoplastic growth, decreased or inhibited foci and/or colony formation (see, e.g., pages 35-36, paragraph [0087]; page 37, paragraph [0090]; and pages 55-58, paragraphs [0138]-[0139]).

The dominant negative nucleic acid binding protein (e.g., a dominant negative protein to Fos) can be expressed throughout the body of the transgenic non-human animal/mammal (e.g., mouse), or in specific tissues by way of a tissue-specific promoter. The specification describes numerous acceptable promoters, including regulated and tissue-specific promoters, at, for example, pages 33-34, paragraphs [0083]-[0084]. Additionally, the specification describes additional regulatory elements, such as enhancers (see, e.g., pages 32-33, paragraph [0081]; pages 34-35, paragraph [0085]; and pages 36-37, paragraph [0089]); expression vectors (see, e.g., pages 32-33, paragraph [0081]); the construction of expression vectors (see, e.g., page 35, paragraph [0086]); and the introduction of the transgene into cells (see, e.g., pages 34-35, paragraph [0085]; and pages 37-40, paragraphs [0091]-[0096]). Moreover, the specification provides numerous examples of specific constructs (e.g., for creation of transgenic non-human animals/mammals, such as mice), as well as describes the successful creation of a transgenic mouse using the methods of the present invention (see, e.g., page 52, paragraph [0130]; page 53, paragraph [0131]; pages 66-69, paragraphs [0159]-[0166]; and Figures 2, 6, and 23). Additionally, the specification provides screening methods to

determine expression of the dominant negative nucleic acid protein (e.g., a dominant negative protein to Fos), such that one of ordinary skill in the art would be able to determine whether a transgenic non-human animal/mammal (e.g., mouse) created using the methods of the invention successfully expressed the transgene (see, e.g., pages 35-36, paragraphs [0087]-[0088]).

Thus, one of ordinary skill in the art would have recognized that Applicants had invented and adequately described a transgenic non-human animal/mammal (e.g., mouse) containing a gene encoding an expressible dominant negative protein to a naturally occurring cellular protein (e.g., a dominant negative protein to Fos), as well as methods of creating the transgenic non-human animal/mammal. For the above reasons, the written description rejection is improper and should be withdrawn.

B. Enablement Requirement

The Office contends that the state of the art of creating transgenic animals was unpredictable at the filing date of the application, such that one of ordinary skill in the art would not know how to make and use the transgenic animal and for what purposes (see Office Action, page 12).

As discussed above, the specification adequately describes and enables the creation of a transgene construct by describing the individual components (e.g., promoters, enhancers, and other regulatory elements), expression vectors and the construction thereof, introduction of the transgene into cells, specific transgene constructs, screening methods to ascertain transgene expression, as well as the successful creation of a transgenic non-human animal/mammal (e.g., mouse) using the methods of the present invention. Additionally, the Rule 132 Declaration of Charles R. Vinson, Ph.D. submitted with the "Response to Office Action" dated March 3, 2004 describes the successful creation of transgenic mice expressing a dominant negative, acidically modified Fos protein (A-FOS) using the methods of the present invention. The specification describes that the transgenic mouse can be used to evaluate and assess the *in vivo* effects of the expression of a dominant negative protein (e.g., a dominant negative protein to Fos) (e.g., in specific tissues and/or in a multi-stage carcinogenesis model), for rational drug design, and for testing supplemental treatments, drugs, and therapies for use with the dominant negative protein (e.g., a dominant negative protein to Fos) (see, e.g., pages 7-8, paragraph [0022], and the Rule 132 Declaration of Charles R. Vinson, Ph.D.). Supplying an inactivating or inhibitory function by the dominant negative protein (e.g., a dominant negative protein to Fos) can lead to and/or cause the suppression of neoplastic growth in a variety of cell types, especially those cells that have been growth-altered (e.g., in a multi-stage carcinogenesis model) (see, e.g., page 37, paragraph [0090] and the Rule 132 Declaration of Charles R. Vinson, Ph.D.). Accordingly,

the specification describes multiple phenotypes associated with dominant negative nucleic acid binding proteins (e.g., a dominant negative protein to Fos) including decreased cell growth, suppression of neoplastic growth, decreased or inhibited foci and/or colony formation (see, e.g., pages 35-36, paragraph [0087]; page 37, paragraph [0090]; and pages 55-58, paragraphs [0138]-[0139]). Furthermore, given the state of the art at the effective time of the filing of the application, one of ordinary skill in the art would have been aware of experimental conditions under which the described phenotype (e.g., decreased cell growth, suppression of neoplastic growth, decreased or inhibited foci and/or colony formation) of a transgenic animal/mammal expressing a dominant negative protein to Fos would have been observed (e.g., during multi-stage carcinogenesis as described in, e.g., Hennings et al., *Carcinogenesis*, *14*(11), 2353-2358 (1993 (attached hereto)). Therefore, given the teachings in the specification, one of ordinary skill in the art would have recognized how to make and use the transgenic mouse of the present invention, as well as for what purpose.

The Office contends that the disclosure of the creation of the transgenic mouse expressing the dominant negative 3heptadF C/EBP in Example 14 is not relevant to the presently claimed invention (e.g., that there is no evidence that indicates the expressible dominant negative 3heptadF C/EBP of Example 14 is an effective dominant negative protein to the Fos protein (see page 12 of the Office Action)). However, the creation of the transgenic mouse expressing the dominant negative 3heptadF C/EBP demonstrates that it is possible to produce a dominant negative nucleic acid binding protein that functions in accordance with the invention in an *in vivo* environment in an animal (see page 68, paragraph [0163]). Additionally, the Rule 132 Declaration of Charles R. Vinson, Ph.D. reports on the successful creation of transgenic mice expressing a dominant negative, acidically modified Fos protein using the methods recited in the specification.

The Office also contends that the disclosure of the A-FOS transgenic mouse in the Rule 132 Declaration of Charles R. Vinson, Ph.D. is not commensurate to the broad genus of a transgenic non-human animal/mammal and method of making the same (see Office Action, page 17). The A-FOS transgenic mouse was produced using the materials and methods of the present invention as described in the specification. Accordingly, the disclosure of the A-FOS mouse in the Rule 132 Declaration is commensurate with the pending claims of the invention.

The Office contends that apart from the embryonic stem (ES) cell approach known in the art at the time of the filing of the application, the specification fails to provide sufficient teaching or examples demonstrating that a transgenic non-human animal can be generated using any embryonic cell type of the non-human animal (see Office Action, pages 13-14). Additionally, the Office contends that the ES cell technology was limited to mice (see Office

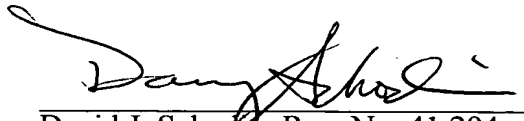
Action, pages 13-14) and that only ES cells and early stage embryos can be used to make transgenic animals (see Office Action, page 17).

The specification describes multiple routes of introducing the transgene into embryonic cells of the transgenic non-human animal/mammal, including transfecting a retrovirus constructed to contain the DNA sequence encoding a dominant negative leucine-zipper containing protein (e.g., Fos) to provide a complete shuttle vector harboring the dominant negative nucleic acid sequence as a transgene and directly injecting a transgene into an embryo, in addition to the use of ES cell technology (see pages 66-67, paragraph [0159]). Indeed, Example 14 describes the introduction of the transgene construct by injection of the construct into early-stage mouse embryos (see, e.g., pages 68-69, paragraph [0164]). The presence of inoperative embodiments within the scope of a claim does not necessarily render a claim nonenabled. The standard is whether a skilled person could determine which embodiments that were conceived, but not yet made, would be inoperative or operative with expenditure of no more effort than is normally required in the art. Applicants have disclosed multiple methods of introducing the transgene into embryonic cells, including the methods of ES cell technology and early stage embryos injection that were known in the art at the time of the filing of the application. For the above reasons, the specification is considered to provide an enabling disclosure, as well as adequate written description, for a transgenic non-human animal/mammal comprising a dominant negative nucleic acid binding protein (e.g., a dominant negative protein to Fos) and method of producing the same.

Conclusion

The application is considered in good and proper form for allowance, and the Examiner is respectfully requested to pass this application to issue. If, in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject application, the Examiner is invited to call the undersigned attorney.

Respectfully submitted,



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FVB/N mice: an inbred strain sensitive to the chemical induction of squamous cell carcinomas in the skin

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The widespread use of FVB/N mice for the establishment of transgenic lines containing active oncogenes suggested the importance of testing the parent FVB/N mice for sensitivity to experimental carcinogenesis. After initiation of mouse skin by a single treatment with 7,12-dimethylbenz[*a*]anthracene (DMBA) and promotion by 20 weekly applications of 12-*O*-tetradecanoylphorbol-13-acetate (TPA), the skin tumor incidence was compared in FVB/N mice, TPA-sensitive (SENCAR and CD-1) and TPA-resistant mice (BALB/c and C57BL/6). Initiation by 25 μg DMBA followed by promotion with a low dose of TPA (2 μg/week) induced one or more papillomas in only 25% of FVB/N mice, compared with 100% in SENCAR, 53% in CD-1, 17% in BALB/c and 0% in C57BL/6 mice. At a more effective dose of TPA (5 μg/week), FVB/N mice initiated by 5, 25 or 100 μg DMBA developed 3.4, 6.9 and 11.8 papillomas per mouse. In contrast, the incidence of squamous cell carcinomas (SCCs) (17–18/30 mice) did not increase with DMBA dose. TPA promotion of non-initiated mice induced only six papillomas, but three progressed to SCCs, a high rate of malignant conversion. Skin tumor induction by 20 weekly treatments with 10 μg DMBA produced few papillomas, but 50.0% of the papillomas progressed to carcinomas in FVB/N mice, compared with 9.15% in SENCAR, 37.5% in CD-1, 23.1% in BALB/c and 15.0% in C57CL/6 mice. The first carcinomas appeared after 14 weeks in FVB/N, 24 weeks in SENCAR, 26 weeks in CD-1 and C57BL/6 and 34 weeks in BALB/c mice. Thus, FVB/N mice develop an unusually high incidence of SCCs after treatment with repeated DMBA, DMBA initiation–TPA promotion and even TPA alone.

Introduction

FVB/N mice, inbred for homozygosity of the allele determining susceptibility to B-type Friend leukemia virus, are widely used for the generation of transgenic mice (1). The advantages of these mice for transgenic studies include: (i) the large, prominent pronuclei of fertilized FVB/N eggs that facilitate the microinjection of DNA; (ii) the large average litter size compared with other inbred strains; and (iii) the detailed genetic characterization, which includes 44 loci on 15 chromosomes.

Several lines of transgenic mice bearing genes with a potential role in neoplasia have been developed using FVB/N mice. Transgenic mice carrying a *mos* protooncogene linked to the Moloney virus long terminal repeat (LTR*) developed both adrenal and thyroid tumors (2). Transgenic mice expressing either the c-*myc* (3) or the c-*neu* (4) oncogene regulated by the mouse mammary tumor virus LTR developed mammary adenocarcinomas, while similarly constructed v-*ras*^{Ha} mice developed adenocarcinomas of the mammary and salivary glands (5). In all three of these transgenic lines, administration of reserpine, a non-mutagenic mammary gland carcinogen, increased the incidence of the oncogene-induced tumors without inducing tumors in other tissues (6). Non-transgenic FVB/N mice treated with reserpine did not develop mammary or salivary gland tumors; no spontaneous tumours of any kind were detected in FVB/N mice (1,6,7). In another line of transgenic mice (designated TG.AC) with an activated v-*ras*^{Ha} oncogene driven by the promoter of the embryonic α-like, ζ-globin gene, skin papillomas developed at areas of epidermal abrasion or after treatment with the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (7).

Carcinogenesis in mouse skin can be divided into three discrete steps, initiation, promotion (8) and malignant conversion (9). Initiation, generally regarded to be a permanent genetic change, is often associated with the activation of the c-*ras*^{Ha} oncogene (10). Promotion, a reversible epigenetic process often accomplished by repeated applications of phorbol esters such as TPA, results in the clonal expansion of initiated cells to produce benign papillomas. Conversion of papillomas to squamous cell carcinomas (SCCs) occurs spontaneously at a low rate, but the rate of malignant conversion can be increased by exposure of papilloma-bearing mice to genotoxic agents. Thus, the conversion mechanism probably involves a post-initiation genetic change.

The TG.AC v-*ras*^{Ha} transgenic mice (7) developed large numbers of papillomas expressing high levels of *ras*^{Ha} mRNA after only 4–6 weeks of TPA exposure. A few papillomas progressed to SCCs, as expected from previous carcinogenesis studies in other strains of mice. Surprisingly, most of the malignant tumors that developed were sarcoma-like, often observed at the base of papillomas. It is unclear whether this unexpected finding was due to (i) TPA-induced expression of the *ras*^{Ha} transgene in fibroblasts, (ii) a unique response of the FVB/N mouse to tumor promoters, unrelated to the transgene, or (iii) a marked anaplastic change in the carcinoma phenotype to yield spindle cell tumors (11).

Leder *et al.* (7) reported that FVB/N mice treated with TPA developed pronounced hyperplasia and hyperkeratosis, but did not develop papillomas. However, the sensitivity of FVB/N mice to the induction of skin tumors resulting from either initiation–promotion or complete carcinogenesis protocols was not determined. We describe here the results of dose–response studies designed to establish effective initiating doses of 7,12-dimethylbenz[*a*]anthracene (DMBA) and promoting doses of TPA in FVB/N mice. Papillomas and an unusually large number of SCCs but no sarcomas, were found. In addition, the responses of FVB/N mice to DMBA initiation–TPA promotion and to repeated treatments with DMBA were compared to

*Abbreviations: LTR, long terminal repeat; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; SCC, squamous cell carcinoma; DMBA, 7,12-dimethylbenz[*a*]-anthracene; K8, keratin 8; GGT, γ-glutamyltranspeptidase; TGF, transforming growth factor; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine.

SENCAR, CD-1, BALB/c and C57BL/6 mice. The highest rate of conversion of papillomas to carcinomas was found in FVB/N mice, suggesting their utility for the study of mechanisms of carcinoma development.

Materials and methods

Chemicals

DMBA was obtained from Eastman Kodak, Rochester, NY; TPA was purchased from LC Services, Woburn, MA. Acetone (99.8% pure), the solvent for DMBA and TPA, was purchased from Curtin Mathesin Scientific, Houston, TX.

Mice

Female mice were purchased at 5 weeks of age. SENCAR outbred, BALB/c, C57BL/6 and FVB/N mice were obtained from the NCI-DCT Animal Program, FCRDC, Frederick, MD. CD-1 mice were purchased from Charles River Laboratories, Wilmington, MA. Five mice were housed in each plastic cage. Mice were shaved at 7–8 weeks of age, 2 days before initiation with DMBA.

Tumor induction experiments

Each experimental group consisted of 25–30 mice. Experiment 1, initiation–promotion with DMBA followed by TPA. Groups of SENCAR, CD-1, BALB/c, C57BL/6 and FVB/N mice were initiated by treating once with 25 μg DMBA and promoted by treating with 2 μg TPA once per week for 20 weeks. This dose of TPA is optimal for promotion in SENCAR mice. Only papillomas were counted, since the experiment was ended at 20 weeks, before carcinomas developed. Neither 'initiation only' nor 'promotion only' controls were included.

Experiment 2, initiation–promotion in FVB/N mice, testing three doses of DMBA and three doses of TPA. Groups of FVB/N mice were initiated with a single application of DMBA (5, 25 or 100 μg), followed by promotion with 20 weekly applications of 5 μg TPA. In other groups, mice initiated by 25 μg DMBA were treated once/week for 20 weeks with either 2 μg TPA, 0.5 μg TPA or acetone solvent. 'Initiation-only' controls were treated with 25 or 100 μg DMBA followed by 20 weeks of exposure to acetone. 'Promotion-only' controls were treated with acetone once, followed by 5 μg TPA each week for 20 weeks. Tumor incidence (papillomas and SCCs) was monitored for 54 weeks after initiation. Experiment 3, complete carcinogenesis with repeated DMBA. Groups of SENCAR, CD-1, BALB/c, C57BL/6 and FVB/N were treated once/week with 10 μg DMBA for 20 weeks. Tumor incidence (papillomas and carcinomas) was monitored for 54 weeks.

In all three experiments, skin tumors were counted every 2 weeks, and mice were weighed once per month. A lesion was recorded as a papilloma when it reached a diameter of > 1 mm and was present for 2 consecutive weeks. The papilloma incidence, the percentage of animals with 1 or more papillomas (percentage with papillomas), and the papilloma multiplicity, the number of papillomas per surviving mouse (papillomas per mouse), were calculated each time tumors were counted. The papilloma latent period was defined as the number of weeks until at least 50% of the mice bore 1 or more papillomas. Suspected carcinomas characterized by cratering and ulceration, with elevation of the margins of the tumor, were verified histologically by standard pathological criteria (12). Since pathological evaluation of tumors that appeared to be papillomas was not performed, early carcinomas arising in papillomas would not have been detected; thus the reported carcinoma incidence probably underestimates the actual incidence. The development of the first carcinoma on a mouse generally causes the animal's death within 4–6 weeks, also resulting in a low estimation of the frequency of

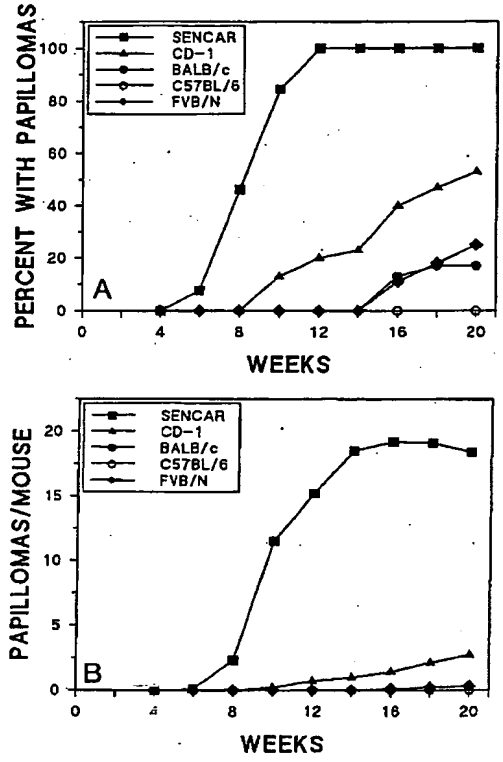

Fig. 1. Initiation–promotion with DMBA–TPA. Groups of 30 SENCAR, CD-1, BALB/c, C57BL/6 and FVB/N mice were initiated by treating with 25 μg DMBA/0.2 ml acetone at zero time and promoted by treating wth 2 μg TPA/0.2 ml acetone once per week from weeks 1–20. Papillomas were counted every 2 weeks and the papilloma incidence and multiplicity were calculated. (A) The per cent with papillomas is plotted versus time (weeks) for the five strains or stocks of mice. The symbols are indicated in the figure. (B) The number of papillomas per mouse is plotted versus time (weeks) for the five strains or stocks of mice. The curves for BALB/c and FVB/N were superimposable and cannot be distinguished.

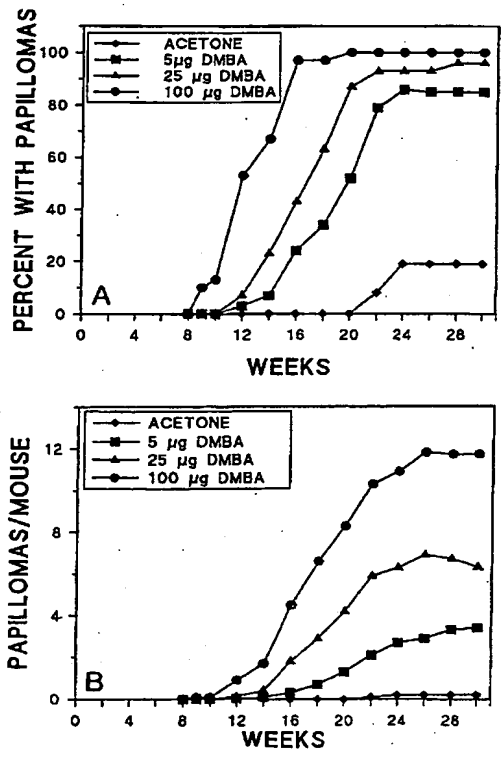

Fig. 2. Initiation–promotion in FVB/N mice: DMBA dose–response. At zero time, mice were treated with either acetone solvent or DMBA (5, 25 or 100 μg/0.2 ml acetone). Mice in all groups were treated with 5 μg TPA/0.2 ml acetone once per week from weeks 1–20. Papillomas were counted every 2 weeks and the papilloma incidence and multiplicity were calculated. This experiment was terminated 54 weeks after initiation; carcinoma data are shown in Table I. (A) The per cent with papillomas is plotted versus time (weeks). The symbols for each treatment group are indicated in the figure. (B) The number of papillomas per mouse is plotted versus time (weeks).

conversion of papillomas to carcinomas (13). The conversion frequency (percentage conversion) was calculated for each group of mice as the ratio of total carcinomas to total papillomas, expressed as a percentage. The carcinoma latent period for each group is expressed as either (i) the number of weeks required for development of the first carcinoma or (ii) the average time for carcinoma development ± SE.

Results

Initiation–promotion: strain comparison

FVB/N mice were compared to two resistant mouse strains, BALB/c and C57BL/6, and two relatively sensitive outbred stocks, SENCAR and CD-1, for susceptibility to DMBA initiation followed by TPA promotion. A single application of 25 μg DMBA was chosen to accomplish initiation in all strains. In order to compare sensitivity to promotion, a low promoting dose of 2 μg TPA weekly for 20 weeks, effective in SENCARs but suboptimal in other strains (14,15), was chosen. The SENCAR mice all developed papillomas by 12 weeks (Figure 1A), with 18.4 papillomas per mouse at 20 weeks (Figure 1B). No tumors were seen in mice of the TPA-resistant strain, C57BL/6. In contrast, papillomas developed in only 53% of the CD-1 mice, 17% of the BALB/c mice and 25% of the FVB/N mice (Figure 1A). Thus, compared with four other stocks and strains of mice, FVB/N mice showed intermediate sensitivity to papilloma induction by this initiation–promotion protocol.

Initiation–promotion in FVB/N mice: dose variation

The susceptibility of FVB/N mice was further characterized by varying the initiating dose of DMBA and the promoting dose of TPA, as described in experiment 2 in Materials and methods. At an effective promoting dose of 5 μg TPA, both the papilloma incidence (Figure 2A) and multiplicity (Figure 2B) increased with dose of the initiator DMBA. The papilloma latent period was ~20 weeks for 5 μg, 16 weeks for 25 μg and 12 weeks for 100 μg DMBA (Figure 2A). Surprisingly, 17–18 SCCs developed in each group of 30 mice (Table I), even though the number of papillomas per mouse varied from 3.4 for 5 μg, 6.9 for 25 μg and 11.8 for 100 μg DMBA (Figure 2B). Consequently, the frequency of conversion of papillomas to carcinomas was 18.5% with 5 μg DMBA, 8.91% with 25 μg, and 5.5% with 100 μg (Table I).

The first carcinoma was seen at 18 weeks with initiation by 100 μg DMBA, 26 weeks with 25 μg DMBA and 32 weeks with 5 μg DMBA (Table I). However, the differences in average carcinoma latent period were much less pronounced (Table I); the only significant reduction was found when mice initiated by 100 μg DMBA were compared with mice initiated by 5 μg DMBA ($P = 0.044$).

In control groups treated with the initiator alone, no tumors developed in mice treated with 25 μg DMBA, and one papilloma developed in mice treated once with 100 μg DMBA. The tumor incidence in mice initiated with 25 μg DMBA and promoted with 2 μg TPA was <1 papilloma/mouse (Figure 1B); no papillomas were seen in mice promoted with 0.5 μg TPA (Table I). However, a group of 26 non-initiated mice treated with 5 μg TPA once weekly for 20 weeks developed six papillomas; three of these progressed to carcinomas by 54 weeks after initiation (Table I). Thus, compared with other stocks and strains of mice, FVB/N mice are not especially sensitive to DMBA initiation or to lower

Table I. Initiation–promotion in FVB/N mice

Group	Number of mice[a]	Initiation[b] [zero time (μg)]	Promotion[c] [weeks 1–20 (μg)]	Papillomas per mouse (maximum)	Total number of papillomas[d]	Total number of carcinomas	Percentage conversion	Carcinoma latent period (weeks)	
								First	Average (± SE)
1	29/27	DMBA (5)	TPA (5)	3.4	92	17	18.5	32	36.5 ± 0.97
2	30/29	DMBA (25)	TPA (5)	6.9	202	18	8.91	26	36.5 ± 1.4
3	30/29	DMBA (100)	TPA (5)	11.8	307	17	5.5	18	32.4 ± 1.7
4	30/NA	DMBA (25)	TPA (0.5)	0	0	0	–	–	–
5	30/NA	DMBA (25)	acetone	0	0	0	–	–	–
6	25/NA	DMBA (100)	acetone	0.04	1	0	–	–	–
7	26/25	acetone	TPA (5)	0.2	6	3	50	38	43.7 ± 3.2

[a]Number initially/number when first carcinoma appeared. NA = not applicable.
[b]0.2 ml of solution in acetone was applied to each mouse's back once at zero time.
[c]0.2 ml of solution in acetone was applied to each mouse's back once per week from weeks 1–20.
[d]The maximum number of papillomas during the 54 week experiment.

Table II. Complete carcinogenesis with repeated DMBA: strain comparison

Group	Mice	Number of mice[a]	Treatment[b] [weeks 1–20 (μg)]	Total number of papillomas[c]	Total number of carcinomas	Percentage conversion	Carcinoma latent period (weeks)	
							First	Average (± SE)
1	SENCAR	30/29	DMBA (10)	153	14	9.15	24	26.7 ± 0.8
2	CD-1	30/30	DMBA (10)	16	6	37.5	26	36.4 ± 3.7
3	BALB/c	30/29	DMBA (10)	13	3	23.1	33	35.5 ± 2.0
4	C57BL/6	29/29	DMBA (10)	40	6[d]	15.0	26	33.6 ± 2.8
5	FVB/N	30/29	DMBA (10)	34	17	50.0	14	26.0 ± 1.8

[a]Number initially/number when first carcinoma appeared.
[b]0.2 ml of solution in acetone was applied to each mouse's back once per week from weeks 1–20.
[c]The maximum number of papillomas during the 54 week experiment.
[d]An additional nine tumors, scored as probable carcinomas based on gross appearance, displayed only chronic inflammation when examined histologically.

promoting doses of TPA, but the conversion rate of papillomas induced by initiation–promotion or by promotion alone is unusually high.

Complete carcinogenesis with repeated DMBA: strain comparison

It has been recognized for many years (16) that complete carcinogenesis protocols are more effective than initiation–promotion for the induction of carcinomas in mouse skin. Twenty weekly treatments with 10 μg DMBA produced the highest papilloma incidence in SENCAR mice, followed in decreasing order of sensitivity by C57BL/6, FVB/N, CD-1 and BALB/c (Table II). Although C57BL/6 mice developed more papillomas than FVB/N (Table II), papillomas developed earlier in FVB/N mice; papilloma latent periods were 16 weeks for SENCAR, 18 weeks for FVB/N and 22 weeks for C57BL/6 mice (data not shown). The first carcinomas were observed in FVB/N mice after only 14 weeks, in SENCAR after 24 weeks, in CD-1 and C57BL/6 after 26 weeks and in BALB/c mice after 34 weeks. By 54 weeks, 17 carcinomas had developed in FVB/N mice, a conversion frequency of 50.0%. Fourteen carcinomas were seen in SENCAR mice, but the conversion frequency was only 9.15% because of the large number of papillomas. Conversion frequencies were 37.5% for CD-1, 23.1% for BALB/c and 15.0% for C57BL/6 mice.

Discussion

The unusual pattern of skin tumor progression in TG.AC ras^{Ha} transgenic mice (7), with spindle cell sarcomas developing at the base of papillomas, prompted our study of the sensitivity to skin carcinogenesis of mice of the FVB/N parent strain. Since no sarcomas were noted among several malignant skin tumors induced in FVB/N mice, the sarcomas observed by Leder *et al.* (7) apparently resulted from cell-specific expression of the ras^{Ha} transgene, rather than from a predisposition of FVB/N mice for sarcoma development. On the other hand, FVB/N mice displayed a high incidence of SCCs after treatment with various tumor induction protocols.

Initiation–promotion

Dose–response studies with FVB/N mice established effective protocols for the induction of skin tumors by initiation with DMBA and promotion with TPA. Initiation by 5 μg DMBA and promotion by 5 μg TPA/week for 20 weeks resulted in 92 papillomas and 17 SCCs in 29 mice. This 18.5% frequency of malignant conversion (Table I) is high compared with the frequency in other strains of mice (14,15). With initiation by higher doses of DMBA, increasing numbers of papillomas were seen, but the carcinoma incidence did not increase. A similar phenomenon (high conversion frequencies associated with initiation–promotion protocols that produced a low papilloma incidence) has been reported in SENCAR mice. The development of 'high risk' papillomas, with high conversion frequencies, results from (i) low initiating doses of DMBA (17,18) (Table I), (ii) promotion with TPA for a short duration (13), or (iii) promotion by 'weak' promoters such as mezerein (13) or chrysarobin (19). 'Low risk' papillomas are produced by higher initiating doses of DMBA or long-term TPA promotion.

Promoter alone

In the group of 26 FVB/N mice treated only with a promoting dose (5 μg) of TPA for 20 weeks, six papillomas developed by week 24. Without further treatment, three of these papillomas progressed to SCCs, noted at 42, 44 and 52 weeks. The autonomy and apparently high conversion frequency of these tumors that developed in the absence of carcinogen treatment suggests that FVB/N mice may possess an inherent predisposition for developing SCCs. In a similar larger experiment with 175 SENCAR females exposed to 5 μg TPA (twice/week for 16 weeks, once/week from weeks 17–45) (15), 53 papillomas developed on 36 mice (not shown). Only five of these tumors progressed to malignancy, a conversion rate of 9.4%. An experiment with larger numbers of FVB/N mice will be required to determine an accurate conversion frequency, but the 50% frequency found here suggests that while SENCAR and FVB/N mice are about equally susceptible to the development of papillomas after exposure of uninitiated mice to the promoter TPA, the potential for malignant conversion of the papillomas induced in FVB/N mice is considerably higher.

Complete carcinogenesis

After repeated DMBA treatment, the first carcinoma was observed in FVB/N mice after only 14 weeks of treatment, 10–12 weeks earlier than SENCAR, CD-1 or C57BL/6, and 20 weeks earlier than in BALB/c mice (Table II). Although SENCAR mice developed 4–5 times as many papillomas, FVB/N mice developed more SCCs; the conversion frequency was 50.0% in FVB/N mice, compared with 9.15% for SENCARs. The early and frequent development of SCCs in FVB/N mice treated repeatedly with DMBA suggests an unusual sensitivity to DMBA or a genetic predisposition for malignant skin tumor development.

The relative sensitivity of various stocks and strains of mice to tumor induction protocols reported here (Figure 1, Table II) is consistent with earlier reports (15,20–22). Differences in metabolism of polycyclic hydrocarbons could determine sensitivity (23), but, in most studies, differences in carcinogen metabolism, DNA binding or formation of specific adducts have not correlated with strain sensitivity (22,24–26). Genetic sensitivity of mouse skin to initiation–promotion protocols has generally been attributed to sensitivity to promotion (27). Although metabolism of phorbol ester tumor promoters is not required for their activity, the effectiveness of promoter treatment could depend on strain-specific responses or on the rate of promoter degradation in the skin. However, comparison of sensitive and resistant mouse strains indicated no differences in phorbol ester binding to protein kinase C (21) or in esterase-induced promoter degradation (22). Neither carcinogen metabolism nor biochemical effects of promoters that may be relevant to promotion have been studied in FVB/N mice.

We have recently established that independent, persistent papillomas induced in SENCAR or CD-1 mice by protocols producing a high conversion frequency can be distinguished from low risk papillomas (28). Papillomas at high risk for progression and conversion to malignancy express the α6/β4 integrin complex suprabasally, display the focal expression of keratin 8 (K8) and γ-glutamyltranspeptidase (GGT), and did not express transforming growth factor (TGF)-β1 or TGF-β2. On the other hand, most low risk papillomas expressed the α6/β4 integrin on the basal surface of basal cells, expressed TGF-β1 and TGF-β2, but did not express either K8 or GGT. These and other markers of keratinocyte differentiation that vary in expression during papilloma development and conversion to malignancy are being investigated in FVB/N tumors. The high conversion frequency in FVB/N mice suggests that they develop few low risk papillomas. Most of the papillomas induced in FVB/N mice may possess the markers characteristic of high risk papillomas and SCCs in SENCAR mice.

Using parent mice other than FVB/N, transgenic mice have been constructed that express the ras^{Ha} (29) or TGF-α gene (30)

in the skin, as directed by the promoter region of keratin genes expressed in the basal or suprabasal layers. With TGF-α overexpression in the basal layer, transgenic mice developed papillomas at sites of abrasion or after wounding or TPA treatment. These papillomas, which did not contain activating mutations in the ras^{Ha} gene, all regressed in the absence of a promoting stimulus. None progressed to malignancy. The ras^{Ha} transgenic mice constructed with ras^{Ha} expression in the suprabasal layers of the skin developed benign papillomas and keratoacanthomas by 10 weeks of age, primarily at sites of mechanical irritation. None of these papillomas converted to carcinomas, perhaps because of the relatively short lifespan of the transgenic mice or the suprabasal origin of the tumors. The TG.AC ras^{Ha} transgenic mice (7) also developed papillomas at sites of skin abrasion or after TPA treatment. While a few SCCs developed, the majority of the malignant tumors were spindle cell sarcomas. From these transgenic studies, neither the TGF-α gene nor the ras^{Ha} oncogene appear to be closely associated with the development of SCCs, although both genes appear to have a role in papilloma development. The papillomas that developed are of the low risk type, with a low rate of progression to carcinomas.

Studies of tumors initiated by treatment of mice with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) provide further evidence suggesting that activating ras^{Ha} mutations may characterize low risk papillomas (31). After MNNG initiation and TPA promotion, activating ras^{Ha} mutations were present in 11 of 15 papillomas, but only 2 of 13 SCCs. Another, as yet undefined, genetic change appears to be critical for development of high risk papillomas.

Introduction of the v-*fos* oncogene into epidermal cells containing an activated ras^{Ha} oncogene produces cells with a malignant phenotype (32,33). When these cells were grafted to the backs of nude mice, SCCs developed. Since the v-*fos* oncogene and the v-*jun* oncogene encode proteins involved in the regulation of gene expression by the AP-1 complex, quantitative changes in AP-1 levels may be critical for malignant conversion. A number of proteases associated with malignancy and metastasis, including stromelysin, collagenase I and urokinase (34–36), are regulated by AP-1 activity. Furthermore, the c-*fos* promoter is downregulated by the tumor suppressor genes *Rb* and *p53*. The future study of these and other relevant genes in FVB/N tumors may lead to an understanding of the sensitivity of FVB/N mice to the development of SCCs.

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